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The Hotis Test for the Detection of Mastitis Bacteria in Milk

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INTRODUCTION

In bovine mastitis, as in other infectious diseases, a satisfactory diagnostic test should be easy to apply and possess a high degree of accuracy. A number of tests for mastitis have been described, but so far none of them entirely fulfill these requirements. The reason is found in the diverse nature of the causes of the disease. Unlike many other infectious diseases, mastitis may occur from invasion of the udder by a number of types of bacteria.

Among the types that are known to cause the disease are streptococci, staphylococci, and several kinds of bacilli. As a rule but one species is present, but mixed infections sometimes occur. Of chief importance are the streptococci. Next in importance are the staphylococci. However, there is some evidence that staphylococcal mastitis may be more widespread in dairy herds than has been supposed. *Aerobacter aerogenes*, *Corynebacterium pyogenes*, and *Pseudomonas aeruginosa* can cause mastitis, usually of an acute nature, but their occurrence is comparatively rare.

Although several species of streptococci are found in the disease, *Streptococcus agalactiae* is the most prominent. It is said to occur in 80 to 90 percent of the cases of chronic mastitis caused by streptococci. A persistent infection of the udder is established as a rule. So far as is known, infection with this species can appear in the herd only through introduction of an infected animal. The other species of streptococci that account for the remaining 10 to 20 percent of the cases do not usually cause persistent infection, nor is their origin known. They may appear in the udder at any time and cause typical

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chronic or acute mastitis, irrespective of whether cases of similar infection are or are not present in the herd. After variable periods of time, ranging from a few weeks or months to more than a year in some cases, these streptococci leave the udder for no apparent reason.

As in the case of the streptococci, several species of staphylococci are encountered in mastitis. Only *Staphylococcus aureus*, however, is of material significance. This species is responsible for a chronic form of the disease that may persist for extended periods, although there is some tendency for the infection to disappear from the quarter spontaneously. Staphylococcal mastitis assumes its most serious form following parturition when an acute attack may terminate fatally. Little is known of the source of the infection, but it may appear in a quarter at any time, even in the absence of other infected animals in the herd. In many of these cases the infection is transitory, but at times a large number of quarters may become permanently infected. Because of the lack of information as to its source, control of the infection is difficult and no specific treatment for it is available at present.

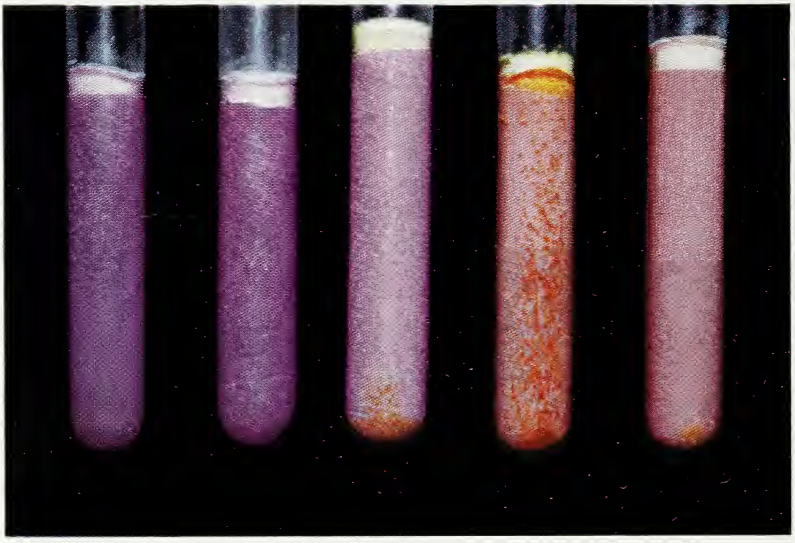
Since chronic mastitis is by far the most prevalent form, several types of tests have been developed for its detection. One of these reveals physical and chemical changes in the milk; a second detects changes in the udder tissue; and a third indicates the kind of bacteria in the milk. The last-mentioned type includes such bacteriological tests as direct culture of unincubated milk samples, direct microscopic examination of incubated milk, and the Hotis test. Definite identification of the bacterial flora in a milk sample is highly desirable but can be accomplished only by direct isolation and pure culture study. Unfortunately this procedure is too cumbersome for use on large numbers of milk samples. On the other hand, both the Hotis test and the microscopic examination of incubated milk can be applied readily to the routine examination of any number of samples, but neither test reveals the exact identity of the offending organisms.

EFFICACY OF THE HOTIS TEST

When it was first described in 1936, the Hotis test[1] was intended as a rapid means of detecting *Streptococcus agalactiae* in milk. At that time investigation of mastitis dealt almost exclusively with this species as the cause of the disease. Since then the importance of other species in this disease has been recognized. A number of reports on the efficacy of the test in the detection of *S. agalactiae* have appeared in the literature. Some investigators reported about 90-percent agreement between results with the Hotis test and those with culture of the sample on blood-agar plates. Other workers found the agreement to be somewhat low, between 30 and 50 percent. The latter reports have been in the minority, however. Since the test has been in use at the Department's Animal Disease Station, Beltsville, Md., 10,000 to 15,000 milk samples have been examined. Each sample was subjected to the Hotis test and culture on blood-agar plates. Compared with the latter, the Hotis test has maintained an average agreement of 85 to 90 percent in detecting cows infected with *S. agalactiae*. Other species of mastitis streptococci are not detected so readily.

[1] HOTIS, R. P., and MILLER, W. T. A SIMPLE METHOD FOR DETECTING MASTITIS STREPTOCOCCI IN MILK. U. S. Dept. Agr. Cir. 400, 7 pp., illus. 1936.

A *B* *C* *D* *E*



F *G* *H* *I* *J*

Appearance of samples after incubation: *A*, *F*, Negative tests—color remains unchanged, no flake formation; *B*, *C*, *D*, and *E*, characteristic of the several changes typical for *Streptococcus agalactiae*—acid formation and few to many yellow or brown flakes; *G*, purple column, white flakes (diphtheroids); *H*, *I*, slightly acid, rust-colored flakes *(Staphylococcus aureus)*; *J*, slightly acid, yellow sediment, no flakes (nonhemolytic staphylococci and streptococci other than *S. agalactiae*).

The tendency of *S. agalactiae* to grow in long chains and to ferment lactose vigorously is probably responsible for the typical changes that permit its recognition. However, other species of streptococci with similar growth habits can produce the same changes. As an example, group G streptococci[2] were encountered in a dairy herd in which mastitis was prevalent. A large percentage of milk samples from this herd were positive when tested by this method. Because of their infrequent occurrence, these types do not affect the results of the test materially.

Of the other streptococci found in mastitis, *S. dysgalactiae* and *S. uberis* are next in importance. That only a small percentage of milk samples containing these two species are positive by the Hotis test probably is due to their growth in short chains and comparatively weak fermentation of lactose. No truly characteristic change is produced by either species, although certain changes encountered in the test may be considered suspicious of their presence. On the other hand, *Staphylococcus aureus* is responsible for a readily identifiable change. These changes, some of which are illustrated in plate 1, were described by Murphy[3] and the bacteria associated with some of them identified.

Failure of the Hotis test to detect the presence of streptococci in all the milk samples containing these organisms has been a source of some criticism. Under the circumstances this is not a serious objection to the method. The principal purpose in developing tests to detect cows with mastitis and its cause, is to control the spread of infection or to indicate cows to be treated. *Streptococcus agalactiae* is the species with which these procedures are primarily concerned, and the test reveals a high percentage of these infections. Other species of streptococci are not susceptible to control by the usual method and frequently leave the udder spontaneously. Their detection is therefore not a pressing problem. Similarly, since no therapeutic or control measures are available for *Staphylococcus aureus*, recognition of the changes produced by it is of value only for differentiation.

TECHNIQUE OF THE TEST

Certain precautions must be observed in carrying out the Hotis test. After careful disinfection of the udder and teats, the milk must be drawn directly into a sterile tube or bottle, preferably one with a small mouth. In the laboratory, all glassware, pipettes, and tubes, as well as the brom-cresol-purple solution, should be sterilized. If the milk is to be transferred from the original container to a test tube for incubation, this must be done as aseptically as possible to avoid contamination. Contaminating bacteria, such as large spore-bearing rods, may multiply rapidly during incubation and digest the milk. Alkali produced during this process may neutralize any acid formed by lactose-forming bacteria and obscure both acid production and the appearance of flakes. Other extraneous bacteria, such as lactic-acid-producing strains, may also interfere with the occurrence of a true positive reaction or even contribute to a false positive reaction.

[2] MILLER, W. T., and HEISHMAN, J. O. BOVINE MASTITIS CAUSED BY UNUSUAL TYPES OF STREPTOCOCCI. Cornell Vet. 30: 310–318. 1940.

[3] MURPHY, J. M. THE VALUE OF THE HOTIS TEST IN DETECTING MASTITIS STREPTOCOCCI IN MILK. Cornell. Vet. 29: 279–286. 1939.

The method therefore cannot be used in examining milk from cans or similar receptacles.

In the test itself, 9.5 cc. of milk is mixed with 0.5 cc. of sterile 0.5-percent brom-cresol-purple solution in a sterile test tube. The outside diameter of the test tube should be not more than 15 mm. The sample is incubated at 37° C. for 20 to 24 hours and the results noted. A longer period of incubation does not improve the results materially and may allow small numbers of contaminating bacteria to increase sufficiently to interfere with the test. When the milk and dye are mixed, a purple color occurs. The reason for this color is that normal milk is very slightly acid (pH 6.3 or slightly over) and this pH is in the upper range of the dye (pH 5.2, yellow to pH 6.8, purple). When a number of tests are set up, however, considerable variation in color may be observed in different tubes. Since the pH of normal milk is slightly acid, these samples will be indicated by a pale grayish-purple or dove color. Samples of milk from quarters affected with mastitis, on the other hand, are more alkaline (pH 6.8 or higher) and have a distinct to deep-purple shade. By noting the color at the time the milk and dye are mixed, as suggested by Cone and Grant,[4] considerable information can be obtained about the physical condition of the quarter or udder. When used in this manner, the Hotis test serves both as a chemical and a bacteriological method.

To avoid the action of contaminating bacteria in obscuring positive changes in the test, certain agents have been added to the milk or the dye. Their function is to inhibit the growth of the contaminants without interfering with the growth of the streptococci. One agent that has been recommended for this purpose is sodium azide. Equal parts of a 0.2-percent aqueous solution of sodium azide and a 0.5-percent solution of brom-cresol-purple are combined and 0.5 cc. of the solution added to 9.5 cc. of milk. Comparative tests with and without sodium azide at the Animal Disease Station show that the growth of contaminants is greatly repressed for at least 48 hours, whereas the streptococci continue to grow. However, the changes are not entirely typical in all cases, and 48 hours' incubation may be required for positive results to occur. In addition, staphylococci do not grow well in the presence of sodium azide. Consequently, some information that might be obtained from the test is lost when sodium azide is used.

SIGNIFICANCE OF REACTIONS TO THE TEST

Milk drawn aseptically from the quarter into a sterile tube usually contains bacteria of some kind. Accordingly, the various changes that occur in milk samples tested by the Hotis method depend on the kind and number of bacteria present. A thorough understanding of the significance of the different changes is therefore a prerequisite to the intelligent use of the test. The more common changes that occur in the Hotis test are illustrated in plate 1.

In this plate, *A* and *F* show the typical appearance of samples giving a negative reaction to the test. The color of the columns of milk remains unchanged after 24 hours' incubation. *B*, *C*, *D*, and

[4] Cone, J. F., and Grant, F. M. Improvement of the Hotis test for the detection of mastitis streptococci. Jour. Milk Technol. 3 (2) 75-79 1940.

E show the characteristic changes produced by *Streptococcus agalactiae*. In a positive sample (one that gives a positive reaction to the test), the color of the column of milk varies from dove gray and olive drab to canary yellow. Flakes or clumps that adhere rather firmly to the side of the tube are always present, as well as coarse flocculent sediment in the bottom of the tube. The sediment may extend a short distance up the side. Both the color of the column of milk and the size and number of flakes appear to depend on the number of streptococci in the milk. Small numbers of organisms cause less change in the color of the milk owing to lower acidity and a small number of large flakes. *B* and *C* are examples of this. A large number of streptococci produce more acid, as evidenced by the definite yellow color of the milk and numerous small flakes (*E*).

The color of the flakes in the positive samples varies somewhat. This variation seems to be characteristic of the several types of streptococci that are designated as *S. agalactiae*. Serologically this species belongs in Lancefield's group B, but several types as well as subtypes have been demonstrated within it. Culturally these are much alike but certain differences are observed among them. These differences are more marked in their growth on blood agar than elsewhere. On this medium, hemolysis varies from none to the production of double beta zones. That these differences in type are reflected in some variation in the appearance of the changes in the positive Hotis tests is illustrated in the upper half of the plate. The flakes in *B* and *D* are brown, whereas those in *C* and *E* are yellow. The brown flakes were produced by a strain identified as a double beta zone streptococcus of group B.[5] The color and appearance of the flakes in the other tubes are typical of those commonly found in positive tests and were caused by a weakly beta hemolytic strain of *S. agalactiae*. Ordinarily the flakes are yellow with a narrow white margin or a small white center. In *E* the white color is somewhat accentuated owing to the large number of flakes. The presence of only one or two typical flakes in a tube is considered positive even in the absence of color changes in the milk.

The changes depicted in the lower half of the plate are frequently observed in the Hotis test and may cause some confusion in interpreting the results of the test. In *G*, the column of milk is unchanged in color but a number of small round white flakes are present on the side of the tube. A somewhat similar appearance is caused by cream sticking to the side of the tube, but differentiation can be made by the ragged appearance of the particles of cream. Diphtheroids (*Corynebacterium lypolyticum*) are usually found in these samples, but all milk samples containing these organisms do not show the flakes. Streptococci that produce small quantities of acid are occasionally responsible for these flakes. In some instances *Streptococcus dysgalactiae* (group C) has been observed to produce them with practically no change in the pH of the milk.

The rust-colored colonies in *H* and *I* are always indicative of *Staphylococcus aureus*. There is a great deal of variation in the ability of different strains of this staphylococcus to ferment lactose. The staphylococci in these two samples were weak acid producers; consequently, there is comparatively little change in the color of the

[5] Personal communication from J. Howard Brown, Professor of Bacteriology, Johns Hopkins University.

column of milk. When strains producing more acid are present, the milk assumes a more definite yellow shade and the flakes are more yellow in color. The center of the flake is yellow or white and the wide outer border is rust colored. At times only one or two small flakes may be present, and a careful examination of the bottom of the tube will be required to find them. An infrequent contaminant that finds its way into milk develops chocolate-colored flakes on the side of the tube. These can be readily differentiated from the flakes of the staphylococci by the color. All milk samples containing staphylococci do not show the characteristic rust-colored flakes, but when they are present they are definitely diagnostic of this infection.

J shows a change that may cause the reaction to be confused with the positive one. The column of milk is usually unchanged or slightly acid. A bright-yellow sediment that may extend a short distance up the side is present in the bottom of the tube. The consistency of the sediment is finely granular for the most part. Occasionally bright-yellow flakes are found on the side of the tube, but as a rule these shake loose easily and fall to the bottom. This reaction is distinguished from a positive one by the difference in the character of the sediment, general absence of flakes on the side of the tube, and the lower acidity. It usually results from the presence of weakly hemolytic staphylococci (*St. epidermidis*). A somewhat similar change may occur when short-chain streptococci that produce little acid are present. The difference in appearance is not sufficiently marked, however, to distinguish between the species.

Several other changes that are observed infrequently should be mentioned. The first of these is a solid-yellow column usually accompanied with coagulation of the milk and possibly some extrusion of whey. Flakes are never present. Bacteria that ferment lactose vigorously are usually present in the milk sample. An olive-green column of milk with an admixture of gas bubbles is observed when small gas-producing rodlike bacteria are present. Large spore-bearing bacilli and similar contaminants cause digestion of the milk with a clear purple supernatant fluid and purplish-gray column of milk below. Finally, a deep-purple color results when the dye is added to abnormal udder secretion or to secretion from a nonlactating udder. Irrespective of the kind of bacteria present, no characteristic changes will occur when such secretion is tested. There is small likelihood, however, that samples manifesting any of the above-mentioned changes will be confused with positive samples.

With proper understanding of the changes that have been described and careful technique, the Hotis test affords a simple and reasonably accurate means of diagnosing the principal causes of bovine mastitis. No elaborate or expensive equipment is required, and a number of milk samples can be tested in a comparatively short time. The latter is an important feature when repeated tests must be made. To determine whether control and treatment of bovine mastitis can be undertaken successfully requires some knowledge of the cause of the disease. Furthermore, when these measures are instituted, repeated examination of milk samples is necessary as a check on their efficacy. Proper use of the Hotis test supplies this information readily and inexpensively.

SUMMARY

The Hotis test of milk as a means of detecting mastitis was originally intended as a rapid means of detecting *Streptococcus agalactiae*, which was regarded as the principal species of bacteria causing the disease. Since the test was first described, in 1936, the importance of other species in this disease has been recognized. Furthermore, although early studies in the Bureau showed very close agreement between the Hotis test and the blood-agar method, a few other workers later reported that their findings showed a somewhat low agreement. Consequently, additional studies have been made at the Department's Animal Disease Station, at Beltsville, Md., on the efficacy of the Hotis test for detecting the various mastitis bacteria. The test consists in mixing, in a sterile tube, 0.5 cc. of sterile 0.5-percent brom-cresol-purple solution with 9.5 cc. of milk carefully collected from the animal. The sample is incubated at 37° C. for 20 to 24 hours.

The studies, involving 10,000 to 15,000 milk samples, showed the test to be 85 to 90 percent as effective as cultures on blood-agar plates for detecting *Streptococcus agalactiae* in milk. This species was detected more readily than other species that cause the disease. Only a small percentage of milk samples containing *S. dysgalactiae* and *S. uberis* are positive by the Hotis test. *Staphylococcus aureus*, which is the most important of the staphylococci causing the disease, is readily identified. This circular describes and illustrates some of the more common changes resulting from *Streptococcus agalactiae* and *Staphylococcus aureus*. These two species can be distinguished by differences in color of samples and of flakes in the milk.

Since *Streptococcus agalactiae* is the usual cause of mastitis and since the form of the disease caused by this species is the only one on which information on control and treatment is available, failure of the Hotis test to detect other species is not a serious objection.

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